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(54) Title: TREATMENT OF MOTOR NEURON DISEASES WITH FIBROBLAST GROWTH FACTOR-5 (FGF-5)

(57) Abstract

The present invention relates to methods of treatment of motor neuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease) comprising administering, to a subject in need of such treatment, an effective amount of a neurotrophic factor termed fibroblast growth factor 5 (FGF-5), which supports the survival, growth and/or differentiation of motor neurons. A combination of FGF-5 and a second agent, preferably CNTF, may be also used in the treatment of motor neuron disease. Also disclosed are methods of promoting the survival, growth and/or differentiation of motor neurons *in vitro*, methods of diagnosis, and assay systems which may be used to identify agents which have FGF-5-like activity and which may be useful in the treatment of motor neuron diseases. The present invention additionally provides pharmaceutical compositions comprising FGF-5 alone or in combination with a second agent which supports the survival, growth and/or differentiation of motor neurons, and a pharmaceutically acceptable carrier.

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**TREATMENT OF MOTOR NEURON DISEASES WITH  
FIBROBLAST GROWTH FACTOR-5 (FGF-5)**

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**1. INTRODUCTION**

The present invention relates to a method of treating a motor neuron disorder comprising 10 administering to a subject in need of such treatment an effective amount of FGF-5 or a functional derivative of FGF-5 which promotes survival of motor neurons. Also provided are methods of culturing motor neurons, methods of diagnosing motor neuron disorders, 15 assay systems for identifying agents which may be beneficial or toxic to motor neurons, and animal models for motor neuron diseases.

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**2. BACKGROUND OF THE INVENTION**

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**2.1. MOTOR NEURONS**

The interaction between motor neurons and muscle cells underlies all voluntary and involuntary body movement by controlling muscle tone and effecting 25 muscular contraction. Motor neurons are traditionally classified as upper motor neurons or lower motor neurons. The cell bodies of upper motor neurons reside in the precentral gyrus of the brain, sending long processes down to form synapses with lower motor 30 neurons in the ventral (anterior) horns of the grey matter of the spinal cord. From the ventral horns of the spinal cord, axon processes of lower motor neurons coalesce to form the ventral roots. These axons eventually terminate on one or more muscle fibers. 35 Through terminal arborization of its axon, each lower motor neuron contacts anywhere from a few up to 100-

200 or more muscle fibers to form a "motor unit," (Adams and Victor, 1985, *PRINCIPLES OF NEUROLOGY*, McGraw-Hill, Inc., New York, p. 37).

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#### 2.2. MOTOR NEURON DISORDERS

Motor neuron disorders result in varying degrees of muscle weakness, causing disabilities ranging from minor deficits in the performance of complex tasks to 10 total paralysis. Lower motor neuron disorders are generally associated with flaccid paralysis and decreased muscle tone. Contiguous groups of muscles, innervated by single nerves or whose motor neurons lie close together in the spinal cord may be affected, and 15 atrophy may be quite profound, including up to about 70-80% of the total muscle bulk.

A diseased motor neuron may also become irritable, leading to sporadic discharge of muscle fibers under its control in isolation from other 20 units. This results in a visible twitch, or fasciculation.

In contrast, damage to upper motor neurons generally results in spastic paralysis, with increased muscle tone and hyperactive tendon reflexes. Rather 25 than individual muscle groups, an entire limb or one-half of the body is usually affected. Muscle atrophy typically resulting from disuse is slight and fasciculations are absent. The identification of a clinical syndrome as representing an upper or lower 30 motor neuron disorder may facilitate its diagnosis and management.

A wide array of neurological disorders affect 35 motor neurons. Upper motor neurons are predominantly affected by cerebrovascular accidents, neoplasms, infections or traumatic injury. Lower motor neurons, or anterior horn cells (AHC), while secondarily

affected by the above processes, are also subject to a number of disorders primarily featuring AHC loss. Such disorders include amyotrophic lateral sclerosis (ALS), infantile and juvenile spinal muscular atrophy, poliomyelitis, the post-polio syndrome, hereditary motor and sensory neuropathies and toxic motor neuropathies, such as that caused by vincristine therapy.

10 Of these predominantly AHC disorders, ALS, also known as Lou Gehrig's disease, is the most common. (For review, see Williams et al., 1991, *Mayo Clin. Proc.* 66:54-82). The initial complaint in most ALS patients is weakness, more commonly of the upper limbs (Gubbay et al., 1985, *J. Neurol.* 232:295-300; Vejjajiva et al., 1967, *J. Neurol. Sci.* 4:299-314; Li et al., 1988, *J. Neurol. Neurosurg. Psychiatry* 51:778-784). Usually the pattern of weakness, atrophy, and other neurological signs is asymmetric and often focal 15 (Munsat et al., 1988, *Neurol.* 38:409-413). Muscle cramps, paresthesia (tingling) and pain are frequent, and widespread fasciculations are usually present. The rate of progression of ALS varies among 20 individuals and almost always results in complete incapacity, widespread paralysis, including 25 respiratory paralysis, and ultimately, death.

The most prominent anatomic changes in ALS are atrophy of the spinal cord and associated ventral roots, and firmness of the lateral columns, hence the 30 name, amyotrophic lateral sclerosis (Williams et al., *supra*). Upper motor neurons also become involved and undergo degeneration. While, the brain may appear normal macroscopically, atrophy of the motor and premotor cortices is usually present due to upper 35 motor neuron involvement. There is widespread loss of Betz cells and other pyramidal cells from the

precentral cortex, with consequent reactive gliosis (Hammer et al., 1979, *Exp. Neurol.* 63:336-346).

5 Epidemiologic studies have indicated that genetic as well as environmental factors play a role in ALS development. Once believed to be a disease of middle-age, it has recently been appreciated that ALS is more a disease of the elderly (Williams et al., *supra*).

10 Current treatment of ALS consists of symptomatic therapy to diminish muscle cramps, pain, and fatiguability. Prosthetic devices compensate for muscle weakness. Pharmacotherapy to alter the progress of the disease has, however, been largely unsuccessful. Putative therapeutic benefits of 15 thyrotropin releasing hormone have met with conflicting results (Brooks, 1989, *Ann. N.Y. Acad. Sci.* 553:431-461). Administration of gangliosides has been ineffective (Lacomblez et al., 1989, *Neurol.* 39:1635-1637). Plasmapheresis has shown no 20 therapeutic advantage either alone and in combination with immunosuppressive treatment (Olarte et al., 1980, *Ann. Neurol.* 8:644-645; Kelemen et al., 1983, *Arch. Neurol.* 40:752-753). The antiviral agent guanidine was reported to have potential short-term benefits, 25 but the results were not reproducible (Munsat et al., 1981, *Neurol.* 31:1054-1055). In an abbreviated study, administration of branched-chain amino acids to activate glutamate dehydrogenase was reported to slow the rate of decline of patients (Plaitakis et al., 30 1988, *Lancet* i:1015-1018). More recent therapeutic trials have involved (a) whole-body total lymphoid irradiation, (b) the use of amino acids N-acetyl-cysteine, N-acetylmethionine and L-threonine, and (c) long-term intrathecal infusion of thyrotropin 35 releasing hormone (Williams et al., *supra*).

Several animal models bear clinical and pathologic resemblance to ALS. The *Mnd* mouse, an autosomal dominant mutant, exhibits late-onset 5 progressive degeneration of both upper and lower motor neurons (Messer et al., 1986, *J. Neurogen.* 3:345-355). The *wobbler* mouse exhibits forelimb weakness and atrophy in early life due to muscle denervation. Hereditary canine spinal muscular atrophy in the 10 Brittany spaniel is also considered an ALS model (Sack et al., 1984, *Ann. Neurol.* 15:369-373; Sillevis et al., 1989, *J. Neurol. Sci.* 91:231-258; Bird et al., 1971, *Acta Neuropathol.* 19:39-50).

15                   2.3. TARGET-DERIVED NEUROTROPHIC FACTORS

During the course of embryonic development, many neuronal populations undergo a period of naturally-occurring cell death. In vertebrates, neuronal 20 survival during this period depends upon access of the developing neurons to specific trophic factors supplied by target tissues. For review, see Oppenheim, *Annu. Rev. Neurosci.* 14:453-501 (1991). For example, nerve growth factor (NGF), produced in limiting 25 quantities in the target tissues of peripheral sympathetic and sensory neurons, supports the survival of these cells during critical stages of development. For review, see Barde, Y.-A., *Neuron* 2:1525-1534 (1989). Similarly, motor neurons rely upon their 30 target during the embryonic period of naturally-occurring cell death (Hamburger, V., *Amer. J. Anat.* 102:365-410 (1958); Hollyday et al., *J. Comp. Neurol.* 170:311-320 (1976)).

Extracts of skeletal muscle, the target of motor 35 neurons, promote the survival of motor neurons in culture (Bennet et al., *Brain Res.* 190:537-542 (1980);

Schnaar et al., *J. Neurosci.* 1:204-217 (1981); Calof et al., *Devel. Biol.* 106:195-210 (1984); Kaufman et al., *J. Neurosci.* 5:160-166 (1985); Flanigan et al., 5 *J. Neurochem.*, 45:1323-1326 (1985); Smith et al., *J. Cell Biol.* 101:1608-1621 (1985); Dohrmann et al., *Dev. Biol.* 118:209-221 (1986); O'Brien et al., *J. Neurosci.* 6:3265-3274 (1986); Martinou et al., *Neuron* 8:737-744 (1989); Arakawa et al., *J. Neurosci.* 10:3507-3515 (1990); Bloch-Gallego et al., *Development* 111:221-232 (1991); Jeong et al., *Neurobiol.* 22:462-474 (1991)). However, attempts to determine the molecular character of the factor or factors involved in regulating motor neuron survival during development have been largely 15 unfruitful.

The present inventors' laboratory demonstrated that several neurotrophic and growth factors support the survival of spinal motor neurons in highly-enriched cultures taken from embryonic day six (E6) 20 chick (Arakawa et al., *supra*). The most active of these, ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF), supported the survival of approximately 50-60% of the initially-plated motor neurons after 3 days in culture. In 25 combination, CNTF and bFGF promoted 100% survival of motor neurons under the *in vitro* conditions employed.

Recombinant CNTF exogenously applied to a newborn rat prevented the otherwise extensive motor neuron degeneration occurring after lesion of the facial 30 nerve (Sendtner et al., *Nature* 345:440-441 (1990)). Such treatment with CNTF rescued a significant number of spinal motor neurons which would otherwise undergo naturally-occurring cell death in the embryonic chick (Oppenheim et al., *Science* 251:1616-1618 (1991)). 35 However, neither CNTF nor bFGF are likely to be

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responsible for the physiological regulation of embryonic neuronal survival, because:

5 (1) both appear to be cytosolic molecules (Abraham et al., *Science* 233:545-548 (1986); Lin et al., *Science* 246:1023-1025 (1989), Stöckli et al., *Nature* 342:920-923 (1989)); and

10 (2) CNTF mRNA is not detectable in skeletal muscle during the period of embryonic motor neuron death in the rat (Stöckli et al., *J. Cell Biol.* 115:447-459 (1991)).

15 To meet the requirements of a neurotrophic factor that regulates motor neuron survival during the embryonic period of cell death, a putative candidate molecule should support motor neuron survival and be appropriately expressed in target tissues.

#### 2.4. FIBROBLAST GROWTH FACTORS AND THEIR ACTIVITIES

20 Fibroblast growth factors (FGFs) comprise a family of potent mitogens for both normal diploid fibroblasts and established cell lines (Gospodarowicz, D. et al., *Proc. Nat'l. Acad. Sci. USA* 81:6963 (1984)). The FGF family comprises acidic FGF, basic FGF, INT-2 (FGF-3), K-FGF/HST (FGF-4), FGF-5, FGF-6, KGF (FGF-7), AIGF (FGF-8) and possibly others. FGFs are also mitogenic for a wide variety of normal diploid mesoderm-derived and neural crest-derived cells, including granulosa cells, adrenal cortical cells, chondrocytes, myoblasts, corneal and vascular endothelial cells (bovine or human), vascular smooth muscle cells, and lens epithelial cells.

30 35 Basic FGF (bFGF) is multifunctional, stimulating proliferation and either inducing or delaying differentiation. The mechanisms action of FGF are

only beginning to be understood (Rifkin, D.B. et al., *J. Cell Biol.* 109:1-6 (1989)). bFGF acts as a differentiation factor for various cells, including 5 chondrocytes, sheep preadipocyte fibroblasts, astrocytes, and oligodendrocytes; bFGF treatment of neural cells induces both neurite outgrowth and ornithine decarboxylase activity. Not all of the effects of bFGF on cell differentiation are 10 stimulatory. For example, bFGF can delay differentiation and fusion of normal diploid myoblasts. FGF also significantly delays the ultimate senescence of various types of cultured cells. When maintained in the absence of bFGF, these cells have a 15 limited life-span, whereas in its presence, they can proliferate for more than 200 generations. In development, bFGF promotes limb regeneration in lower vertebrates, and may play a role in the early development of the nervous system. Basic FGF promotes 20 both the survival and differentiation of nerve cells derived either from the hippocampus or the cortex. FGFs may also have pronounced effects on the proliferation and differentiation of astrocytes and oligodendrocytes during normal development or 25 following a specific pathogenic event.

Due to the widespread distribution of bFGF, and its broad range of target cells, numerous names have been used to designate this molecule. FGF has been used for the treatment of ischemic heart disease 30 (Franco, U.S. Patents 4,296,100 and 4,378,347), increasing blood flow in the heart for sustained periods after myocardial infarction. Nevo et al. (U.S. Patent No. 4,642,120) disclosed the use of FGF for repairing defects of cartilage and bones. Senoo 35 et al. (European Patent Publication EP281822)

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disclosed a mutein of bFGF which accelerated cell growth *in vitro* and acted as a healing accelerator for burns and a therapeutic drug for thrombosis. Arakawa 5 et al., European Patent Publication EP320148) disclosed recombinant bFGF analogs with at least one of the biological properties of mammalian bFGF including the induction of neovascularization, re-epithelialization and wound repair. bFGF stimulates (a) 10 growth of bone marrow stromal cultures, which are capable of supporting hemopoietic cell growth and differentiation, and (b) myelopoiesis in bone marrow culture (Oliver, L.J. et al., *Growth Factors* 3:231-236 (1990); Wilson, E.L. et al., *Blood* 77:954-960 (1991)).

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#### 2.5. FGF-5

A protein originally termed FGF-3, but now designated FGF-5, was discovered as the gene product of an oncogene called *ORF-2* (Goldfarb, M. et al., PCT Publication WO 88/01767, Dec. 1, 1988; see, also, 20 Zhan, X. et al., *Oncogene* 1:369-376 (1987); Zhan et al., *Mol. Cell. Biol.* 8:3487-3495 (1988); these three references are hereby incorporated by reference in their entirety). The name "FGF-3" was selected 25 because of the substantial homology to the two already described FGFs (aFGF and bFGF). By the time the sequence was publicly disclosed (Zhan et al., 1988, *supra*), two additional FGF-related polypeptides, INT-2 and HST/K-FGF, had already been described. Hence, 30 FGF-3 was redesignated FGF-5. To avoid confusion, the name FGF-5 is used herein. Goldfarb et al. (*supra*) showed that the amino acid sequences of two segments of FGF-5 (residues 90-180 and 187-207) shared 40.2% homology with aFGF and 50.4% homology with hst/KS3

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but was nevertheless substantially different from other members of the FGF family.

The structure of the rearranged transforming FGF-5 oncogene was similar to other FGF-related genes. The FGF-5 gene was expressed in a human brain stem cDNA library, and several human tumor cell lines contained transcripts which hybridized with an FGF-5 cDNA probe. Transfection of an FGF-5 cDNA clone into NIH-3T3 cells resulted in transformants which expressed the FGF-5 oncogene and secreted a growth factor apparently encoded by this gene. This secreted growth factor bound to heparin, a property shared with aFGF and bFGF. The presumed FGF-5 product also resembled partially purified bFGF in its stimulation of endothelial cell growth. Finally, extracts of bacteria expressing the FGF-5 protein contained a heparin-binding growth factor with properties similar to those of conditioned medium from FGF-5-transfected eukaryotic cells.

The FGF-5 protein has 268 amino acids (corresponding to the second open reading frame or "ORF 2" as disclosed in Goldfarb et al., *supra*) the sequence of which is well-conserved across mammals. The murine homologue shows 84% overall sequence identity to the human (Hébert et al., *Dev. Biol.* 138:454-463 (1990)). Unlike the prototypical aFGF and bFGF, FGF-5 contained a hydrophobic N-terminal leader sequence typical of a secreted protein (Goldfarb et al., *supra*) and was released into the medium of transformed NIH-3T3 cells (Bates et al., *Mol. Cell. Biol.* 11:1840-1845 (1991)). Mouse embryos prior to gastrulation contain mRNA encoding FGF-5, suggesting that this gene product has a function during very early embryonic development (Hébert et al.,

*Development* 112:407-415 (1991); Haub et al.,  
*Development* 112:397-406 (1991)).

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#### 2.6. CILIARY NEUROTROPHIC FACTOR AND MOTOR NEURON SURVIVAL

10 Ciliary neurotrophic factor (CNTF) promotes the survival of motor neurons *in vitro* and *in vivo* (PCT Publication No. WO 91/04316, 4/4/91, incorporated by reference herein). Gelfoam implants containing CNTF facilitated survival of motor neurons in severed facial nerves of newborn rats. CNTF is a neurotrophic factor that exhibits biological activities that are 15 very different from those exhibited by FGF-5, as described herein.

#### 3. SUMMARY OF THE INVENTION

20 The present inventors have discovered that FGF-5 promotes the survival of motor neurons *in vitro* and is therefore useful for promoting motor neuron growth and survival. On this basis, it is expected that FGF-5 is useful *in vivo* for treating a disease associated with 25 a deficiency in motor neuron number or function.

30 The present invention relates to a method of treating a motor neuron disorder comprising administering to a subject in need of such treatment an effective amount of FGF-5, or a functional derivative thereof that supports the survival of motor neurons.

35 The present invention is further directed to a method of treating a motor neuron disorder comprising administering to a subject in need of such treatment an effective amount of FGF-5, or a functional

derivative thereof that supports the survival of motor neurons, in combination with an effective amount of at least one other agent that is capable of promoting 5 motor neuron survival, growth, or differentiation. Preferably the other agent is ciliary neurotrophic factor. The other agent may be bFGF or a member of the brain-derived neurotrophic factor/ neurotrophin-3/ nerve growth factor (BDNF/NT-3/NGF) family of 10 molecules, that supports the survival, growth and/or differentiation of motor neurons.

The above methods are useful for treating any motor neuron disorder such as those associated with trauma, amyotrophic lateral sclerosis, progressive 15 spinal muscular atrophy, infantile muscular atrophy, juvenile muscular atrophy, poliomyelitis, post polio syndrome, or Charcot-Marie-Tooth disease.

The present invention is also directed to a pharmaceutical composition useful in the treatment of 20 a motor neuron disorder comprising;

- (a) an amount of FGF-5, or a functional derivative thereof, effective in treating a motor neuron disease or disorder; and
- (b) a pharmacologically acceptable carrier.

In another embodiment, the pharmaceutical 25 composition comprises, in addition to FGF-5 or a derivative thereof, at least one other agent that is capable of promoting motor neuron survival, growth, or differentiation. A preferred other agent is ciliary 30 neurotrophic factor.

Also provided is a method of promoting motor 35 neuron survival, either *in vivo* or *in vitro*, comprising exposing a motor neuron to an effective concentration of FGF-5 that is capable of promoting the survival of motor neurons.

In another embodiment, the present invention is directed to a method of diagnosing a motor neuron disorder associated with an abnormal level of FGF-5 in a subject, comprising:

- 5 (a) measuring the level of FGF-5 in a sample from the subject;
- (b) comparing the levels of FGF-5 in step (a) with the level of FGF-5 in an analogous sample from a 10 normal individual or with a standard level of FGF-5.

thereby detecting any abnormality in the level of the FGF-5 in the subject, the abnormality being diagnostic of the motor neuron disorder. The disorder is 15 preferably selected from among amyotrophic lateral sclerosis, progressive spinal muscular atrophy, infantile muscular atrophy, poliomyelitis, post-polio syndrome, Charcot-Marie Tooth disease, nerve trauma or nerve injury.

20 Also included is a method for assaying a test agent for its ability to alter the binding of FGF-5 to a motor neuron, comprising:

- 25 (a) exposing a motor neuron to detectably labeled FGF-5 under conditions wherein the labeled FGF-5 binds to the motor neuron;
- (b) exposing the motor neurons to the test agent;
- (c) measuring the binding of FGF-5 to the motor neurons in the presence of the test agent;
- (d) exposing the motor neurons to a control agent;
- 30 (e) measuring the binding of FGF-5 to the motor neurons in the presence of the control agent; and
- (f) comparing the binding of the labeled FGF-5 in the presence of the test agent with the binding of labeled FGF-5 in the presence of the control agent,

wherein an increase or decrease in the binding of labeled FGF-5 to the motor neurons in the presence of the test agent indicates that the agent alters the 5 binding of FGF-5 to a motor neuron.

The present invention is also directed to a method for assaying a test agent for its ability to enhance or inhibit the activity of FGF-5 in promoting the survival of motor neurons, comprising:

- 10 (a) exposing motor neurons to a concentration of FGF-5 effective in promoting the survival of the neurons;
- (b) exposing the motor neurons to the test agent;
- (c) measuring the survival of the motor neurons in the presence of the test agent;
- 15 (d) exposing the motor neurons to a control agent;
- (e) measuring the survival of the motor neurons in the presence of the control agent; and
- (f) comparing the survival of the motor neurons in 20 the presence of FGF-5 and the test agent with the survival of the motor neurons in the presence of FGF-5 and the control agent.

The present invention further provides for animal models of motor neuron disease produced by immunizing 25 an animal to FGF-5 or derivatives thereof or by administering to an animal anti-FGF-5 antibodies or antisense oligonucleotides specific for FGF-5.

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#### 4. DESCRIPTION OF THE DRAWINGS

Figure 1 shows Northern blots of RNA hybridized with a 500 bp cRNA probe to FGF-5. Panel A: 16  $\mu$ g of total RNA from embryonic (E15 and E17), newborn (NB) 35 and adult (A) rat skeletal muscle. Blot exposed for 7

days. Panel B: Comparison of the size of the FGF-5 transcript in 5  $\mu$ g total RNA obtained from E15 rat skeletal muscle (E15) and adult rat hippocampus (H).  
5 Blot exposed for 3 days. The positions of the FGF-5 specific message and the 18S and 28S ribosomal RNA bands are indicated by arrows.

Figure 2 is a graph showing the effects of increasing concentration of recombinant human FGF-5 on 10 survival of cultured E6 chick motor neurons in the absence (○) and presence (●) of heparin (500 ng/ml). Results are means  $\pm$  1 standard deviation from a minimum of 4 cultures from at least 2 independent experiments.

15 Figure 3 is a graph showing a comparison of maximum motor neuron survival activity and synergism of FGF-5 with CNTF and other members of the FGF family. Factors were added to cultures in concentrations previously determined to promote 20 maximal survival: FGF-5, aFGF, hst/K-FGF - 100 ng/ml; bFGF - 20 ng/ml; CNTF - 1 ng/ml. Results are means from 6 determinations from three independent experiments. Error bars represent 1 standard error of the mean (SEM). \* Significantly different survival 25 compared to cultures containing bFGF alone,  $p < 0.00005$ . \*\* Significantly different from cultures containing CNTF alone,  $p < 0.005$  (Student's t-test).

Figure 4 is a graph showing motor neuron survival activity of increasing concentrations of recombinant 30 human FGF-5 prior to (●) or following immunoprecipitation with either anti-FGF-5 (○) or "control" anti-CNTF (□) serum.

Figure 5 is a graph showing motor neuron survival activity of E17 muscle extracts. Column A: Control, 35 no muscle extract. Columns B-D: Addition of 250  $\mu$ g/ml

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PBS-soluble extract prior to (column B) and following immunoprecipitation with anti-FGF-5 (column C) or anti-CNTF (column D) antisera. Columns E-G: Addition of 70  $\mu$ g/ml high molar salt/EDTA extract prior to (column F) or following immunoprecipitation with anti-CNTF (column G) serum. Results are means from at least 4 cultures from at least two independent experiments. Error bars represent 1 SEM.

\* Significantly different from PBS soluble extract prior to immunoprecipitation,  $p<0.0005$ .

\*\* Significantly different from high salt extract prior to immunoprecipitation,  $p<0.00005$  (Student's t-test).

Figure 6 is a graph showing motor neuron survival activity of adult muscle extracts. Column A: Control, no muscle extract. Columns B-D: Addition of 125  $\mu$ g/ml PBS-soluble extract prior to (column B) and following immunoprecipitation with anti-FGF-5 (column C) or anti-CNTF (column D) sera. Columns E-G: Addition of 7  $\mu$ g/ml high molar salt/EDTA extract prior to (column E) and following immunoprecipitation with anti-FGF-5 serum (column F) or anti-CNTF serum (column G). Results are means from 4 cultures from two experiments. Error bars represent 1 SEM.

\* Significantly different from PBS soluble extract prior to immunoprecipitation,  $p<0.05$ .

\*\* Significantly different from high salt extract prior to immunoprecipitation,  $p<0.0005$ .

\*\*\* Significantly different from high salt extract prior to immunoprecipitation,  $p<0.05$ . (Student's t-test).

5. DETAILED DESCRIPTION OF THE INVENTION5.1. FGF-5 AS A TROPHIC FACTOR FOR SPINAL  
MOTOR NEURONS

5 The present inventors have discovered that FGF-5 is a major survival factor, present in skeletal muscle, for cultured spinal motor neurons and plays a role in the maintenance of motor neuron survival 10 during development and in adulthood. Because of this activity, FGF-5 is useful in methods for promoting survival of motor neurons and in the treatment of diseases and disorders associated with loss of functional motor neurons.

15 In a preferred embodiment, FGF-5, as described in Goldfarb, M. et al., PCT Publication WO 88/01767, Dec. 1, 1988, hereby incorporated by reference in its entirety) is used to promote the survival of motor neurons.

20 In another embodiment, a naturally occurring mammalian FGF-5 is used. In another embodiment a recombinant mammalian FGF-5 is used. In yet another embodiment, a chemically synthesized mammalian FGF-5 protein or glycoprotein is used. Methods for the 25 synthesis of polypeptides of desired sequence on solid phase supports and their subsequent separation from the support are well-known in the art.

30 The preferred FGF-5 protein or glycoprotein useful in the methods and compositions of the present invention is of human origin.

The naturally occurring FGF-5 protein or glycoprotein used according to the present invention is preferably substantially free of other proteins or glycoproteins with which it is natively associated.

35 "Substantially free of other proteins or

glycoproteins" indicates that the FGF-5 protein or glycoprotein has been purified away from at least 90% (on a weight basis), and from even at least 99%, if 5 desired, of other proteins and glycoproteins with which it is natively associated, and is therefore substantially free of them. Such purification can be achieved by subjecting cells, tissue or fluid containing FGF-5 to a standard protein purification 10 technique, for example, immunoaffinity chromatography using an immunoadsorbent column to which is immobilized a monoclonal antibody (mAb) which binds to the protein. Alternatively, or additionally, FGF-5 glycoprotein is purified using a combination of 15 standard methods, such as ammonium sulfate precipitation, molecular sieve chromatography, heparin affinity chromatography and ion exchange chromatography. It will be understood that a mammalian FGF-5 protein or glycoprotein of the present invention 20 can be biochemically purified from a variety of cell or tissue sources. For preparation of a naturally occurring FGF-5 protein or glycoprotein, tissues such as embryonic skeletal muscle, especially of human origin, are preferred. When preparing FGF-5 from 25 adult tissue, it is preferable to use high salt concentration and EDTA to dissociate the FGF-5 (see, for example, Section 6, below). Cell lines producing FGF-5, such as FGF-5-transformed NIH-3T3 cells (Goldfarb, *supra*) may also be used.

30 Because the gene for FGF-5 protein or glycoprotein can be isolated or synthesized, FGF-5 protein can be synthesized substantially free of other proteins or glycoproteins of mammalian origin in a prokaryotic organism or in a non-mammalian eukaryotic 35 organism, if desired. As intended by the present

invention, a recombinant FGF-5 protein or glycoprotein produced in mammalian cells, such as transfected COS, NIH-3T3, or CHO cells, for example, is preferably a naturally occurring protein sequence. Where a naturally occurring FGF-5 protein or glycoprotein is produced by recombinant means, it is provided substantially free of the other proteins and glycoproteins with which it is natively associated.

10

### 5.2. FUNCTIONAL DERIVATIVES OF FGF-5

Also provided herein are methods and pharmaceutical compositions comprising a functional derivative of FGF-5. By "functional derivative" is meant a "fragment," "variant," "analogue," or "chemical derivative" of the protein, which terms are defined below. A functional derivative retains at least a portion of the function of the FGF-5 protein, for example reactivity with an antibody specific for the FGF-5 protein, the ability to bind to spinal motor neurons, the ability to promote growth or survival of spinal motor neurons which evidences its utility in accordance with the present invention.

The term "fragment" is used to indicate a polypeptide which is derived from FGF-5, preferably human FGF-5, and has naturally occurring protein sequence. Such a fragment may be produced by proteolytic cleavage of the full-length protein. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the FGF-5 protein or glycoprotein to delete one or more amino acids at one or more sites of the C-terminal, N-terminal, and within the native sequence.

Fragments of a FGF-5 protein or glycoprotein are also useful for screening for compounds that are antagonists or agonists of FGF-5 action. It is 5 understood that such fragments may retain one or more characterizing portions of the native protein or glycoprotein. A preferred retained characteristic for use according to this invention is the capacity to promote spinal motor neuron growth or survival.

10 Another functional derivative intended within the scope of the present invention is a FGF-5 variant with amino acid deletions, insertions, substitutions or a combination thereof, relative to the naturally occurring protein. Such variants may be derived from 15 a naturally occurring FGF-5 protein or glycoprotein by appropriately modifying the DNA coding sequence to add codons for one or more amino acids at one or more sites of the C-terminal, N-terminal, and within the native sequence. It is understood that such a variant 20 having additional amino acids retains one or more characterizing portions of the native FGF-5 protein or glycoprotein, as described above. A preferred variant is one which has substituted amino acids, the variant being derived from a naturally occurring FGF-5 by 25 appropriately modifying or mutating the DNA coding sequence to substitute one or more amino acids at one or more sites of the C-terminal, N-terminal, and within the native amino acid sequence. It is understood that such a variant having substituted 30 amino acids retains one or more characterizing portions of the native FGF-5.

A preferred group of variants is that in which at least one amino acid residue in the protein molecule, and preferably, only one, has been removed and a 35 different residue inserted in its place. For a

detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions which may be made in the protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al. (*supra*) and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
- 20 5. Large aromatic residues: Phe, Tyr, Trp.

25 The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation which is important in protein folding. Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, etc.

Substantial changes in functional or immunological properties are made by selecting substitutions that are less conservative, such as 5 between, rather than within, the above five groups, which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or 10 hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (a) substitution of Gly and/or Pro by another amino acid or deletion or insertion of Gly or Pro; (b) substitution of a hydrophilic residue, 15 e.g., Ser or Thr, for (or by) a hydrophobic residue, e.g., Leu, Ile, Phe, Val or Ala; (c) substitution of a Cys residue for (or by) any other residue; (d) substitution of a residue having an 20 electropositive side chain, e.g., Lys, Arg or His, for (or by) a residue having an electronegative charge, e.g., Glu or Asp; or (e) substitution of a residue having a bulky side chain, e.g., Phe, for (or by) a residue not having such a side chain, e.g., Gly.

Most deletions and insertions, and substitutions 25 according to the present invention are those which do not produce radical changes in the characteristics of the FGF-5 protein or peptide molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of 30 doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by site-specific mutagenesis of the protein-encoding nucleic acid, expression of the variant nucleic acid 35 in recombinant cell culture, and, optionally,

purification from the cell culture, for example, by immunoaffinity chromatography using a specific antibody on a column (to absorb the variant by binding to at least one epitope).

The activity of the cell lysate or a purified or semi-purified protein variant can be screened in a suitable screening assay for the desired characteristic. For example, a change in the immunological character of the protein peptide molecule, such as binding to a given antibody, is measured by a standard immunoassay. Biological activity is screened in an appropriate bioassay, as described herein. Modifications of such properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct of a FGF-5 functional derivative, provided that the final construct possesses at least one desired activity or function of the intact FGF-5 protein or glycoprotein, as described above.

In another embodiment, a functional derivative of FGF-5 glycoprotein with amino acid deletions, insertions or substitutions (or a combination thereof) may be conveniently prepared by direct chemical synthesis, using methods well-known in the art.

Also included in the present invention is a "chimeric" FGF-5 molecule, constructed from FGF-5 in which one or more specific amino acid sequences are replaced with functionally active sequence(s) from

another protein or peptide. Preferably the sequences are replaced with homologous sequences.

5 A "chemical derivative" of the FGF-5 protein or FGF-5 peptide contains additional chemical moieties not normally a part of the protein. Covalent modifications of the protein or peptide are included within the scope of this invention. Such 10 modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

15 Cysteinyl residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction 20 with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ (5-imidozoyl) propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

25 Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium 30 cacodylate at pH 6.0.

35 Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing

residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; 0-  
5 methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, 10 and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high  $pK_a$  of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the 15 arginine epsilon-amino group.

Modifications of tyrosyl residues are well-known in the art, in particular modifications which introduce spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most 20 commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction carbodiimide ( $R'-N-C-N-R'$ ) such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) 25 carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residue are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are 30 frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this 35 invention.

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Derivatization with bifunctional agents is useful for cross-linking the FGF-5 protein or peptide to a water-insoluble support matrix or to other 5 macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including 10 disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl]dithiolpropioimide yield photoactivatable 15 intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 20 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side 25 chains (Creighton, T.E., *supra*), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the 30 solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's

*Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

5           5.3. BIOLOGICAL ACTIVITIES OF FGF-5

The biological activity of FGF-5 or of a functional derivatives thereof, may be measured as motor neuron growth-promoting activity or 10 differentiation-promoting activity, using any method known in the art. In preferred, non-limiting embodiments, increased survival of motor neurons may be measured by the method set forth in Arakawa et al., *J. Neurosci.* 10:3507-3515 (1990)). Increased 15 sprouting of motor neurons may be detected by methods set forth in Pestronk et al. (1980, *Exp. Neurol.* 70:65-82) or Brown et al. (1981, *Annu. Rev. Neurosci.* 4:17-42). In a preferred embodiment, an assay is 20 performed as described in Section 6, below. Increased production of motor neuron-associated molecules may be measured by bioassay, enzymatic assay, immunoassay, 25 Northern blot assay, etc., depending on the molecule to be measured. Motor neuron dysfunction can be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, defective motor neuron conduction velocity, or functional disability.

In additional embodiments of the invention, FGF-5 may be combined with a second agent in order to support motor neuron survival and/or differentiation. 30 Desirably, the second agent is also effective in promoting motor neuron survival and/or differentiation. For example, in a preferred embodiment of the invention, FGF-5 is combined with CNTF in order to promote motor neuron survival and/or 35 differentiation. As discussed in section 6.2.2,

infra, FGF-5 combined with CNTF exerts at least an additive effect on motor neuron survival.

5 5.4. THERAPEUTIC APPLICATIONS OF FGF-5

10 The present invention provides for methods of treatment of a motor neuron disorder comprising administering, to a subject in need of such treatment, an effective amount of FGF-5, or a functional derivative thereof, that supports the survival, growth or differentiation of motor neurons.

15 As used herein, the term "motor neuron disorder" refers to any condition that disturbs the normal function of motor neurons. Such disorders may or may not specifically or even selectively affect motor neurons. For example, motor neuron disorders that may be treated according to the invention include, but are not limited to, disorders such as infarction, 20 infection, toxin exposure, physical or surgical trauma, degenerative disease or malignant disease that affects motor neurons as well as any other components of the nervous system.

25 The methods of this invention may also be used to treat disorders that selectively affect motor neurons such as amyotrophic lateral sclerosis, and including, but not limited to, progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, 30 progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis, post polio syndrome and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

35 Effective doses of FGF-5 for therapeutic uses discussed above may be determined using methods known

to one skilled in the art. Effective doses, as well as supraoptimal or toxic doses, if they exist, may be determined, preferably *in vitro*, in order to identify 5 the optimal dose range (see section 5.5, below).

FGF-5 may also be administered in combination with an effective amount of at least one other agent that is, itself, capable of promoting motor neuron survival, growth, or differentiation. For example, 10 FGF-5 may be administered with CNTF or with a member of the BDNF/NT-3/NGF family, including but not limited to BDNF, NGF, NT-3, NT-4 and NT5. In a preferred embodiment, FGF-5 and CNTF are co-administered in the treatment of a motor neuron disorder.

15 Since soluble heparin potentiates the survival effect of FGF-5 on motor neurons, another embodiment provides administering FGF-5 in combination with heparin. The preferred dose of heparin is one which achieves a tissue concentration of between about 10 20 ng/ml and 10  $\mu$ g/ml, more preferably about 1  $\mu$ g/ml.

Also included in the present invention is the use of a functional derivative of FGF-5, such as a peptide fragment thereof. Also intended is the use of agonists or antagonists of FGF-5.

25 The FGF-5 or other neurotrophic factor(s) of the invention may be administered in any pharmaceutically acceptable carrier. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, 30 subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

In a preferred embodiment, an aqueous solution of FGF-5 is administered by subcutaneous injection. Each 35 dose may range from about 0.5  $\mu$ g to about 50  $\mu$ g FGF-5

per kilogram body weight, or more preferably, from about 3  $\mu$ g to 30  $\mu$ g FGF-5 per kilogram body weight.

The dosing schedule for subcutaneous administration of FGF-5 may vary from once a week to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the subject's sensitivity to FGF-5. Nonlimiting examples of dosing schedules are 3  $\mu$ g/kg administered twice a week, three times a week or daily; a dose of 7  $\mu$ g/kg twice a week, three times a week or daily; a dose of 10  $\mu$ g/kg twice a week, three times a week or daily; or a dose of 30  $\mu$ g/kg twice a week, three times a week or daily.

Effective doses of additional neurotrophic factors administered in combination with FGF-5, such as CNTF, are in the same dose range as the effective doses of FGF-5 described herein.

If the subject manifests undesired side effects such as temperature elevations, cold or flu-like symptoms, fatigue, etc., it may be desirable to administer a lower dose at more frequent intervals. One or more additional drugs may be administered in combination with FGF-5 to alleviate such undesired side effects, for example, an anti-pyretic, anti-inflammatory or analgesic agent.

Once a desired clinical effect has been achieved, the dose or frequency of FGF-5 administration may be decreased or increased to a "maintenance dose" which maintains the subject's clinical status at a desired level. If a subject develops resistance to FGF-5, the dose may be increased. Clinical effects of FGF-5 therapy may be determined using methods well-known in the art for the particular disease, including tests of nerve function, muscle function, etc. Such tests may

be used to monitor a subject's response to therapy and thereby optimize the dosing schedule during initial and maintenance phases of treatment.

5 In the case of more severe disease, it may be preferable to administer doses such as those described above by alternate routes, including intravenously or intrathecally. Continuous infusion of FGF-5 may also be appropriate.

10 Administration of FGF-5, or of a pharmaceutical composition of the present invention, may result in either systemic or localized distribution of the FGF-5 (and any co-administered neurotrophic factor) depending on the route of administration and the dose.

15 For some conditions which multiple and distant regions of the nervous system, intravenous or intrathecal administration of FGF-5 in place of, or in addition to, subcutaneous administration, may be preferable. Alternatively, and not by way of limitation, when only localized regions of the nervous system are involved, such as in motor neuron disorders caused by trauma or surgery, local administration may be desirable. In such situations, an implant containing FGF-5 (alone or with one or more neurotrophic factors) may be placed in or near the affected area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

20 FGF-5 may also be administered via a cellular, solid or semi-solid implant to achieve blood levels of FGF-5 similar to those attained by subcutaneous administration. A cellular implant may comprise cells naturally producing, or genetically altered to produce, FGF-5, which secrete FGF-5 *in vivo* in a subject following inoculation.

The present invention also provides pharmaceutical compositions comprising an amount of FGF-5 effective to promote motor neuron survival and effective to treat a disease associated with motor neuron dysfunction, in a pharmaceutically acceptable carrier. Also provided is a pharmaceutical composition comprising an effective amount of FGF-5 together with one or more additional neurotrophic factors, preferably CNTF or a member of the member of the BDNF/NT-3/NGF family, in a pharmaceutically acceptable carrier.

#### 5.5. IN VITRO APPLICATIONS OF FGF-5

The present invention provides for a method of promoting motor neuron survival, growth, and/or differentiation comprising exposing motor neurons to an effective concentration of a FGF-5. This method may be carried out *in vivo* (see Section 5.4, above) or *in vitro*.

For use *in vitro*, an effective amount of FGF-5, or a combination of FGF-5 with one or more neurotrophic factors, can be determined on a case-by-case basis, as motor neurons from different tissue sources or from different species may exhibit different sensitivities. For any particular culture or cell type, it is desirable to obtain a dose-response curve that correlates FGF-5 concentration and motor neuron response.

To evaluate motor neuron survival, growth, and/or differentiation, it may be useful to compare motor neurons exposed to FGF-5 to motor neurons not exposed to such factor, using, for example, vital dyes to evaluate survival, phase-contrast microscopy and/or

neurofilament stain to measure neurite sprouting, or techniques that measure the bioactivity of motor neuron-associated compounds, such as the enzyme 5 choline acetyltransferase (CAT).

CAT activity is measured, for example, by harvesting and lysing treated or untreated motor neurons in a 20 mM Tris-HCl (pH 8.6) solution containing about 0.1% Triton X-100, removing an 10 aliquot of several microliters, and measuring CAT activity. Enzymatic activity is determined using, as a substrate, 0.2 ml [1-<sup>14</sup>C] acetyl-CoA, 300 mM NaCl, 8 mM choline bromide, 20 mM EDTA, and 0.1 mM 15 neostigmine in 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) buffer, using the micro-Fonnum procedure as described in Fonnum, 1975, *J. Neurochem.* 24:407-409, incorporated by reference in its entirety herein.

For testing the activity of FGF-5 alone or in combination with another neurotrophic factor *in vitro*, 20 motor neurons may be prepared and cultured as described in the Examples, below, which exemplify chick and rat motor neurons. The skilled artisan will readily appreciate how to use these methods, with or without modification, for the culture of motor neurons 25 of other species, including humans, without undue experimentation.

Chick spinal motor neurons are prepared by first dissecting lateral motor columns from the lumbar region of several E6 chick spinal cords, followed by 30 trypsin treatment and trituration. The resultant cell suspension is filtered and centrifuged through a bed of 6.8% metrizamide. Cells from the interface are collected and the suspension concentrated by centrifugation, resuspended in CO<sub>2</sub>-buffered L-15 medium 35 supplemented with horse serum (10%) and plated,

preferably in 4-well culture dishes (C.A. Greiner und Söhne GmbH, Nürtingen, FRG) at a concentration of about 1000-2000 cells/well. The culture wells are 5 previously coated with poly-DL-ornithine and laminin, the latter solution being removed just prior to cell plating. The cells are incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 1 hour, test factors and/or extracts added at the appropriate 10 concentration, and the cells are returned to the incubator for the length of the experiment. The culture medium and factors may be augmented or replaced after 24 hours. Large phase-bright cells (≥14 µm in diameter) are counted about 72 hours after 15 plating (unless otherwise specified) in predetermined areas corresponding to 23% of each well bottom.

In any of the motor neuron culture systems, such as the two described herein, FGF-5 is added alone or with another one or more neurotrophic factors. 20 Preferred final concentrations of FGF-5 are between about 1 ng/ml and 300 ng/ml, more preferably about 100 ng/ml. FGF-5, at the above concentrations may be added in conjunction with CNTF at a final concentration of at least about 1 ng/ml and preferably about 10 ng/ml. The motor neuron cultures may then be 25 maintained in serum-free defined medium at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere at nearly 100% relative humidity.

30                   5.6.    DIAGNOSTIC APPLICATIONS OF FGF-5

The present invention also provides for a method of diagnosing a motor neuron disorder in a subject, comprising comparing the level of FGF-5 in a sample 35 from the subject with an analogous sample from a

normal individual, or with a standard level previously determined from normal individuals, and thereby detecting an aberrancy in the level of FGF-5 in the 5 subject which positively correlates with the presence of the motor neuron disorder. Motor neuron disorders that may be diagnosed in this manner include but are not limited to those listed above.

The neurotrophic factor level may be detected by 10 any method known in the art including, but not limited to, immunoassays, preferably enzyme-linked immunoabsorbent assay (ELISA), such as a "sandwich"-type ELISA, or any other assay that utilizes an antibody specific for FGF-5, including Western blot analysis and *in situ* hybridization. Also useful are 15 measurements of biological activity of FGF-5 or molecular measurements such as Northern blot analysis, etc.

Samples may be derived from any appropriate 20 tissue or fluid of a subject, including but not limited to, nerve or brain tissue or cerebrospinal fluid or blood.

An aberrancy in FGF-5 level may be defined as a 25 statistically significant difference in the level of FGF-5 in the subject compared to a normal control. The aberrant level may be increased or decreased relative to normal levels, and/or may exist throughout the subject or only in a localized area. Subjects exhibiting a decrease in FGF-5 may particularly 30 benefit from administration of the factor, alone or in combination with another agent which promotes motor neuron growth, differentiation or survival. Subjects exhibiting an increase in neurotrophic factor may be expressing a structurally abnormal FGF-5 or may suffer 35 from an effective paucity of FGF-5 receptors.

Subjects with fewer than normal, or abnormal, FGF-5 receptors may be identified by testing cells of such subjects for their levels or activities of FGF-5 receptor, for example assessing the ability of such cells to bind detectably labeled FGF-5 and comparing the binding to that of cells from a normal, healthy individual. Subjects with fewer or abnormal receptors may or may not benefit from supplemental FGF-5 therapy, and may particularly benefit from treatment with an FGF-5 agonist or analogue.

#### 5.7. ASSAY SYSTEMS

The present invention provides assay systems useful for identifying compounds that can treat motor neuron disorders. Such assay systems evaluate a test agent for the ability to block FGF-5 binding to motor neurons. Accordingly, the present invention provides a method for assaying a test agent for its ability to promote the survival, growth, or differentiation of motor neurons comprising exposing motor neurons in culture to detectably labeled FGF-5 capable of binding to the cells in the presence of the test agent. Competitive inhibition of FGF-5 binding by the test agent indicates that the test agent may be used in the treatment of motor neuron disorders. A test agent identified in such an assay system is preferably further evaluated *in vitro* or *in vivo* for its ability to promote the survival, growth, or differentiation of motor neurons in culture.

FGF-5 may be detectably labeled by any method, including by the incorporation of radiolabelled amino acids, or by the addition of a detectable exogenous label. Such a label may be fluorescent, may have

enzymatic activity, may be an antibody that binds to FGF-5, or may be an antigen capable of binding to a detecting antibody.

5 In one embodiment of this invention, for example, radiolabelled FGF-5 together with an unlabeled test agent are incubated with motor neurons in culture under conditions that allow FGF-5 binding. As a control, an identical motor neuron culture is exposed to radiolabelled FGF-5 in the absence of the test agent. The cells are then washed to remove unbound FGF-5, and the radioactivity associated with the cells is measured. If the test agent binds to the FGF-5 receptor on the neurons, and competitively inhibits 10 binding of labeled FGF-5, cells incubated with the test agent will have less radioactivity than the 15 control cells.

#### 5.8. ANIMAL MODEL SYSTEMS

20 The present invention further provides for animal model systems for motor neuron disorders comprising animals that have systemic or generalized depletion of FGF-5. Generalized depletion of FGF-5 may be achieved by producing an animal that contains or produces 25 antibody directed toward FGF-5. For example, animals are immunized with FGF-5 from another species or with FGF-5 that has been chemically modified to increase its immunogenicity.

Local depletion of FGF-5 may be achieved by 30 introducing anti-FGF-5 antibody locally, e.g., by implanting a hybridoma cell line producing the antibody in a location where it is desirable to deplete FGF-5 activity. Antibodies useful in these animal models are antibodies, polyclonal, monoclonal, 35 chimeric, other others, which neutralize the

biological activity of FGF-5, preferably, its motor neuron growth promoting or survival promoting activity.

5

6. EXAMPLE: FGF-5 IS A MAJOR MUSCLE-DERIVED SURVIVAL FACTOR FOR CULTURED SPINAL MOTOR NEURONS

The present inventors discovered that a member of 10 the FGF family, FGF-5, acts as a physiologically relevant neurotrophic factor and promotes motor neuron survival. These inventors found that recombinant human FGF-5 supported the survival of chick spinal motor neurons in culture and also showed that FGF-5 15 mRNA is present in embryonic skeletal muscle during the period of naturally-occurring motor neuron death as well as in adult muscle. Extracts of these muscles had activity in promoting motor neuron survival. An antibody to FGF-5 removed most of this activity from 20 the muscle extracts. On the basis of these discoveries, the present inventors concluded that FGF-5 is a target-derived trophic factor for spinal motor neurons.

25

6.1. MATERIALS AND METHODS

6.1.1. Materials

Recombinant human FGF-5 was purified from the 30 soluble fraction of *E. coli* lysates by heparin-Sepharose chromatography and Mono-S fluid phase liquid chromatography (FPLC). (See: Goldfarb, M. et al., *supra*, Zhan, X. et al., 1987, *supra*; Zhan et al., 1988, *supra*).

Recombinant rat CNTF was prepared from 35 transfected *E. coli* using a modification of the method

described by Masiakowski et al., *J. Neurochem.*, 57:1003-1012 (1991), hereby incorporated by reference), in which the chromatographic purification 5 of the protein on DEAE cellulose was replaced by a single reverse-phase HPLC step on a semi-preparative, butyl column (Baker). Recombinant human K-FGF was provided by Dr. Claudio Basilico, New York University, New York, USA. Recombinant aFGF and bFGF were gifts 10 from Dr. W. Risau, Martinsried, FRG.

N-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives, 2-(1H-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU), HMP 15 resin, solvents and miscellaneous reagents for peptide synthesis were from Applied Biosystems, Weiterstadt, FRG. Freund's complete and incomplete adjuvants, Leibovitz's L-15 medium were from GIBCO BRL, 20 Eggenstein, FRG. Metrizamide was from Serva, Heidelberg, FRG. General laboratory reagents were from Sigma Chemie, Deisenhofen, FRG and E. Merck, Darmstadt, FRG.

#### 6.1.2. Northern Blots

25 Hindlimb skeletal muscle was dissected from embryonic (E15 and E17), newborn and adult rats killed by ether overdose, taking particular care to exclude bone, cartilage and skin, and total RNA extracted from the muscle according to the method of Chomczynski et al., *Anal. Biochem.* 162:156-159 (1987)). 20 µg of 30 each RNA sample (determined spectrophotometrically) were glyoxylated and electrophoresed through a 1.4% agarose gel (Lindholm et al., *J. Biol. Chem.*, 263:16348-16351 (1988)) and vacuum blotted onto nylon 35 membranes (Hybond-N, Amersham, Braunschweig, FRG).

The membranes were hybridized overnight in 50% formamide as described (Castrén et al., Proc. Natl. Acad. Sci. (1992)) with a 500 bp cRNA probe to FGF-5.

5 The probe (specific activity  $10^9$  cpm/ $\mu$ g) was made by run-on transcription of a pBluescript SK<sup>+</sup> vector containing a 2.2 kb mouse *Fgf-5* cDNA fragment (Haub et al., *supra*) linearized with *Bgl*III.

10 6.1.3. Synthesis of FGF-5 Peptide and Antiserum Preparation

The nonadecapeptide CFKQSEQPELSFTVTVPKEK [SEQ ID NO:1] comprises a relatively hydrophilic 18 amino acid sequence from a C-terminal part of human FGF-5 (Phe<sup>217</sup>-Lys<sup>234</sup>). This sequence is highly conserved between animal species (100% identity between human and mouse) but bears no homology to other members of the FGF family.

15 The above peptide was synthesized on an Applied Biosystems 431A automated peptide synthesizer using N-Fmoc amino acid derivatives and HBTU assisted coupling steps. Following deprotection and cleavage from the resin, the crude peptide free acid was purified as required by reverse phase HPLC from 0.1% TFA on a 20 butyl column using an acetonitrile gradient (0-70% acetonitrile in 30 min.).

25 New Zealand rabbits were immunized by intracutaneous injection into at least four sites on the back with 700  $\mu$ g of the peptide dissolved in 500  $\mu$ l water and emulsified with an equal volume of Freund's complete adjuvant. Rabbits were boosted with a similar quantity of peptide solution emulsified with Freund's incomplete adjuvant at three to four weekly intervals. Blood samples were obtained from an ear 30 vein one week after boosting, allowed to coagulate (at

- 41 -

37°C for 2 hours then overnight at 4°C), centrifuged (at 6000 x g, 25 min.). The resultant serum was sterile filtered (0.2 µm filters) and used in 5 immunoprecipitation assays.

#### 6.1.4. Motor neuron Cultures

Chick spinal motor neuron cultures were prepared 10 as previously described (Arakawa et al., *supra*). Briefly, lateral motor columns were dissected from the lumbar region of six E6 chick spinal cords, 15 trypsinized and triturated. The resultant cell suspension was filtered and centrifuged through a bed of 6.8% metrizamide. Cells from the interface were collected and the suspension concentrated by 20 centrifugation, resuspended in CO<sub>2</sub>-buffered L-15 medium supplemented with horse serum (10%) and plated in 4-well culture dishes (C.A. Greiner und Söhne GmbH, Nürtingen, FRG) at a concentration of 1000-2000 25 cells/well. The wells had been previously coated with poly-DL-ornithine and laminin, the latter solution being removed just prior to plating. The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 30 1 hour, factors and/or extracts added at the appropriate concentration, and the cells returned to the incubator for the length of the experiment. The culture medium and factors were renewed after 24 hours. Large phase-bright cells (≥14 µm in diameter) were counted 72 hours after plating (unless otherwise specified) in predetermined areas corresponding to 23% of each well bottom.

6.1.5. Preparation of Skeletal Muscle Extracts

Hindlimb skeletal muscle was dissected from  
5 embryonic (E17) and adult rats as described above.  
Extracts of soluble skeletal muscle proteins were  
obtained by homogenizing the muscle on ice in two to  
three volumes of PBS in a glass-glass homogenizer and  
centrifugation (100,000  $\times$  g, 4°C, 30 min.).

10 Matrix-bound components were obtained by re-  
extraction of the pellets obtained above with 2 or 3  
initial muscle volumes of PBS supplemented with 3M  
NaCl and 10 mM EDTA, followed by centrifugation as  
above. The protein concentration of the resultant  
15 extracts was determined by the method of Bradford  
(Anal. Biochem. 72:248-254 (1976)) using bovine serum  
albumin as a standard. The skeletal muscle extracts  
were stored at -70°C until use.

20 6.1.6. Immunoprecipitation Experiments

30  $30 \mu\text{l}$  protein G-Sepharose Fast Flow suspension  
was centrifuged (14,000 rpm, 5 min.), and the beads  
were resuspended in 0.5  $\times$  TBS (pH 7.4, 150  $\mu\text{l}$ ). To  
25 this suspension was added 200  $\mu\text{l}$  of either anti-FGF-5  
peptide antiserum prepared as described above or a  
"control" antiserum raised against a 27 amino acid  
peptide from CNTF (Antiserum I, Stöckli et al., 1991,  
supra). The mixture incubated for one hour at room  
30 temperature on an inverting mixer. The protein G  
Sepharose-antiserum complex was washed three times  
with 500  $\mu\text{l}$  TBS, after which 20  $\mu\text{l}$  0.5  $\times$  TBS and  
either (a) 60  $\mu\text{l}$  muscle extract (5  $\mu\text{g}/\mu\text{l}$ ) or (b) 60  $\mu\text{l}$   
35 recombinant human FGF-5 solution (1 ng/ $\mu\text{l}$  in L-15( $\text{CO}_2$ )  
medium supplemented with 10% horse serum) was added.

Following centrifugation, the resultant supernatants were tested for survival promoting activity by adding them to motor neuron cultures.

5

## 6.2. RESULTS

### 6.2.1. FGF-5 mRNA is Expressed in Adult and Embryonic Rat Skeletal Muscle

To determine whether FGF-5 mRNA is expressed in the target tissue of motor neurons either during embryonic development or in adulthood, Northern blot analysis was performed on total RNA obtained from hindlimb skeletal muscle of embryonic (E15 and E17), newborn and adult rats. Following high stringency washing, a cRNA probe from mouse FGF-5 was found to specifically hybridize to a band migrating marginally faster than the 18S band in rat skeletal muscle RNA at all timepoints studied (Figure 1). The probe also hybridized to a band of similar size in RNA prepared from other rat tissues, including adult hippocampus (D. Lindholm, *supra*), a known site of FGF-5 expression in the mouse (Haub *et al.*, *supra*).

25

### 6.2.2. FGF-5 Supports Survival of E6 Chick Spinal Motor neurons in Culture

The ability of recombinant human FGF-5 to support the survival of E6 chick motor neurons *in vitro* was determined in the absence and presence of heparin (Figure 2). Alone, FGF-5 supported the survival of a maximum of 27% of the initially-plated motor neurons at a concentration of 10 ng/ml after 72 hours in culture. In the presence of heparin sulphate (500 ng/ml), the survival effect of FGF-5 was increased significantly, such that 45% of plated motor neurons

were supported at a concentration of 100 ng/ml. Motor neurons cultured in the presence of the same concentration of heparin were not significantly 5 different than control cultures.

The concentration of FGF-5 required to give half maximal survival ( $EC_{50}$ ) of motor neurons was 800 pg/ml. Thus, in this particular culture system, FGF-5 was significantly better than other heparin-dependent 10 members of the FGF family which were tested, aFGF and *hst*/K-FGF (maximally 35% and 32% survival, respectively, 3 days; Figure 3). FGF-5 was less effective in promoting neuronal survival than were either bFGF or CNTF (maximally 50% and 61% survival, 15 respectively, 3 days).

Furthermore, the combination of FGF-5 with either CNTF or bFGF at supramaximal concentrations in the presence of heparin (500 ng/ml) gave additive effects on motor neuron survival. Thus, that FGF-5 plus CNTF 20 supported approximately 74% survival, and FGF-5 and bFGF supported 80% survival (Figure 3).

In contrast to CNTF and bFGF, FGF-5 did not support the survival of cultured E8 chick ciliary ganglion neurons.

25

#### 6.2.3. Antibody to FGF-5 Peptide Reacts with Factor(s) Responsible for Neuronal Survival

To investigate the contribution of FGF-5 to the motor neuron survival activity of embryonic and adult 30 rat skeletal muscle extracts, we performed immunoprecipitation experiments with an antiserum specific for FGF-5.

The antiserum was raised in rabbits by immunization with a synthetic peptide representing a 35 sequence from the C-terminal portion of human FGF-5.

The sequence was judged by hydropathy analysis (Hopp et al., *Proc. Natl. Acad. Sci.* 78:3824-3828 (1981); Kyte et al., *J. Mol. Biol.* 157:105-132 (1982)) to be 5 relatively hydrophilic and therefore likely to be exposed on the surface of intact FGF-5. Furthermore, the peptide spans a region of the C-terminal "tail" of FGF-5 which, although well-conserved between species, is not present in the other known members of the FGF 10 family. This reduced the likelihood that the resultant antibodies would cross-react with other members of the FGF family.

Although antiserum raised against this peptide did not recognize recombinant human FGF-5 on 15 immunoblots, it was possible to precipitate the motor neuron survival activity of recombinant human FGF-5 almost entirely using the antiserum (Figure 4). This finding indicated that the antiserum recognized native FGF-5 in solution. In contrast, this antiserum did 20 not immunoprecipitate the motor neuron survival activity of any other member of the FGF family tested (aFGF, bFGF and *hst/K*-FGF) or of CNTF.

Figure 5 shows the motor neuron survival activity 25 of an extract of phosphate buffered saline- (PBS-) soluble components of embryonic (E17) rat skeletal muscle before (column B) and after (column C) the addition of FGF-5 antiserum which had been immobilized on protein G-Sepharose. Following centrifugation of the antiserum-Sepharose complex, the survival activity 30 of the soluble embryonic muscle extract on E6 chick spinal motor neurons at the maximum protein concentration tested (250 µg/ml) was reduced from 60% to 36%. This represents a 47% reduction in survival activity, given the background survival of 9% in 35 control cultures (Figure 5, column A). This result

suggests that FGF-5 was responsible for a significant proportion of the survival activity of the PBS-soluble components of E17 rat skeletal muscle.

5      Because members of the FGF family, including FGF-5, tend to bind to components of the extracellular matrix (ECM), we tested whether embryonic rat skeletal muscle contained additional, matrix-bound, FGF-5-like motor neuron survival activity which was not  
10     extractable with PBS alone. Thus, the pellets obtained after PBS extraction of E17 muscle were re-extracted with PBS containing 3M NaCl and 10mM ethylenediamine tetraacetic acid (EDTA). These conditions would be expected to disrupt interactions  
15     between ECM and molecules bound to it, allowing solubilization of matrix-bound components.

The high molar salt extract of E17 muscle had a weaker survival promoting effect on cultured chick spinal motor neurons than did the PBS extract  
20     (approximately 37% survival; Figure 5, column E). Notably, this effect was achieved at a lower total protein concentrations (60 µg/ml).

25     Immunoprecipitation of the high salt extract with anti-FGF-5 antiserum reduced by 83% the maximal motor neuron survival of the extract (Figure 5, column F).

30     Thus, motor neuron survival activity extractable from E17 rat skeletal muscle was immunoprecipitable by anti-FGF-5 antiserum, and approximately half of this activity was apparently quite tightly bound to the ECM. "Control" precipitation of either the soluble or matrix-bound extract of embryonic muscle with anti-CNTF antiserum did not significantly reduce motor neuron survival activity (Figure 5, columns D and G).

6.2.4. FGF-5 Protein in Adult Muscle is Matrix-Bound

5 Immunoprecipitation of adult muscle extracts with anti-FGF-5 antiserum suggests that FGF-5 protein is almost entirely matrix-bound.

10 To determine whether FGF-5 was present in adult rat skeletal muscle extracts, anti-FGF-5 antiserum bound to Sepharose was tested for its ability to remove motor neuron survival activity from adult muscle extracts.

15 PBS-soluble extracts of adult skeletal muscle exhibited maximal motor neuron survival activity of approximately 37% at a protein concentration of 125  $\mu$ g/ml (3 day culture; Figure 6, column B). However, unlike the PBS-soluble extract of embryonic muscle, none of the activity could be precipitated with anti-FGF-5 antiserum (Figure 6, column C).

20 Conversely, most (71%) of the maximal survival activity of the high molar salt extract of adult rat skeletal muscle (33% at 7  $\mu$ g/ml protein) was precipitated by the anti-FGF-5 (Figure 6, columns E and F). The finding that FGF-5 immunoprecipitable activity could not be extracted from adult rat skeletal muscle with PBS, but instead required high salt and EDTA indicated that FGF-5 in adult rat muscle was almost exclusively bound tightly to ECM.

25 A small proportion of both the survival activity in PBS-soluble and high-molar salt/EDTA extracts of adult muscle (approximately 30% and 29%, respectively) were immunoprecipitated with anti-CNTF antiserum (Figure 6, columns D and G).

6.3. DISCUSSION

5 The motor neuron survival activity of embryonic and adult rat skeletal muscle appears to exist in two forms: that which was readily-extractable with PBS, and that which required high molar salt and EDTA for solubilization. Of the four types of muscle extract prepared, the most effective on promoting survival of chick motor neurons was PBS-soluble extract of E17 muscle. The remaining preparations had lower, and 10 very similar survival promoting activity. It is possible that the maximal effects of both types of adult extracts were reduced by the presence of toxic components.

15 The higher survival activity found in embryonic compared to adult skeletal muscle may reflect a greater reliance of developing embryonic motor neurons on trophic support during the period of naturally-occurring motor neuron death. Alternatively, adult 20 muscle produces a "neurotrophic cocktail" which is not optimally suited to embryonic neuronal survival.

25 Interestingly, high salt/EDTA extraction of embryonic and adult skeletal muscle yielded preparations having apparent specific activities (relative to total protein content) which were approximately 4-fold and 17-fold greater, respectively, than their PBS-soluble counterparts. Thus, compared to soluble proteins, a larger proportion of matrix-bound proteins in muscle exhibit 30 motor neuron survival activity.

35 A specific antiserum to FGF-5 was used to test whether the motor neuron survival activity of the muscle extracts was attributable to FGF-5. In hindlimb skeletal muscle of E17 rats (an age at which naturally-occurring embryonic motor neuron death is

underway; Oppenheim, R.W., *J. Comp. Neurol.* 246:281-286 (1986)), approximately 60% of the total maximal extractable survival activity was immunoprecipitated by the anti-FGF-5 antiserum. This immunoprecipitable activity was distributed between a PBS-soluble form and a form which required high molar salt and EDTA for extraction. In contrast, FGF-5 activity in adult skeletal muscle was exclusively in the matrix-bound form.

10 FGF-5, like the other members of the FGF family, exhibits strong binding to the glycosaminoglycan heparin (Zhan et al., *supra*) and thus would be expected to be tightly bound to heparin sulphate-like components of the ECM. Thus, the greater resistance to solubilization of the FGF-5 activity from adult muscle compared to embryonic muscle reflects a difference in the nature or composition of the ECM in the adult animal. Similar developmentally-related changes in the ease of extractability of other components of the ECM, such as laminin, are known (Kücherer-Ehret et al., *Development* 10:1285-1293 (1990)).

15 FGF-5 activity in E17 skeletal muscle extracts could originate from two sources: the muscle itself and/or a site of earlier expression. The observed expression of FGF-5 mRNA in this tissue is support for the presence of FGF-5 in muscle. Indeed, in the mouse, the most prominent embryonic expression of FGF-5 mRNA at times beyond gastrulation occurs at E12.5 to E14.5 in a patch of mesenchyme at the base of the hindlimb (Haub et al., *supra*). FGF-5 protein expressed in the developing rat may therefore be deposited in the surrounding muscle, thus contributing 20 to the observed FGF-5-like activity in the E17 muscle

extract. However, the expression of FGF-5 mRNA in adult rat muscle is a strong indication that the muscle itself is the major source of FGF-5 in this 5 tissue. The previously reported absence of FGF-5 mRNA in Northern blots of RNA from adult mouse skeletal muscle (Haub et al., *Proc. Natl. Acad. Sci.* 87:8022-8026 (1990)) suggests either a difference in regulation of the FGF-5 gene in these two species, or 10 that the amount of FGF-5 mRNA in mouse fell below their detection limits. The latter interpretation is supported by the fact that levels of FGF-5 mRNA in adult rat skeletal muscle are lower than in some 15 central nervous system sites, such as the hippocampus (D. Lindholm, *supra*).

An antiserum to a 27 amino acid peptide from CNTF (Stöckli et al., 1989, 1991, *supra*) was selected as a control for the present study. The CNTF antiserum did not remove motor neuron survival activity of 20 recombinant FGF-5 or from embryonic muscle extracts. This is in agreement with observations that rat CNTF mRNA is below detectable limits during the period of naturally-occurring motor neuron death (Stöckli et al., 1989, 1991, *supra*). Based on the above, it is 25 highly unlikely that the action of the anti-FGF-5 antiserum could be attributed to binding of neurotrophic factor(s) to non-specific IgG, which increases following immunization. The small, yet significant, reduction in the survival activity from 30 adult muscle extracts caused by the CNTF antiserum was probably due to neural CNTF in the nerves which innervate adult muscle. Indeed, CNTF immunoreactivity is detectable in Schwann cells surrounding nerves in sections of adult skeletal muscle. Furthermore, in 35 extracts of adult rat skeletal muscle which has been

5 surgically denervated for 7 days, during which time nerve fibers and their accompanying Schwann cells distal to the lesion site degenerate, the motor neuron survival promoting activity is no longer removable by anti-CNTF.

10 A family of membrane-spanning receptor tyrosine kinases, encoded by at least four distinct genes, are capable of acting as receptors and signal-transducing 10 units for FGFs (Ruta et al., *Oncogene* 3:9-15 (1988); Kornbluth et al., *Mol. Cell. Biol.* 8:5541-5544 (1988); Lee et al., *Science* 245:57-60 (1989); Dionne et al., *EMBO J.* 9:2685-2692 (1990); Pasquale, E.B., *Proc. Natl. Acad. Sci. USA* 87:5812-5816 (1990); Miki et al., 15 *Science* 251:72-75 (1991); Keegan et al., *Proc. Natl. Acad. Sci. USA* 88:1095-1099 (1991); Partanen et al., *EMBO J.* 10:1347-1354 (1991); Stark et al., *Development* 113:641-651 (1991)). These tyrosine kinases exist in a variety of molecular forms generated by alternative 20 RNA splicing (Johnson et al., *Mol. Cell. Biol.* 10:4728-4736 (1990); Johnson et al., *Mol. Cell. Biol.* 11:4627-4634 (1991)) and bind different members of the FGF family with differing affinities (Johnson et al., *supra*; Miki et al., *supra*; Partanen et al., *supra*).

25 The binding of FGFs to the receptor tyrosine kinases requires the participation of heparin sulphate proteoglycan or soluble heparin. (For review, see Klagsbrun et al., *Cell* 67:229-231 (1991).) Recombinant human FGF-5 is able to bind and activate 30 FGF receptors 1 and 2 (D. Clements, et al., *Oncogene*, in press, (1993)). The relative affinities of FGF-5 and bFGF towards these receptors correlate with the relative concentrations of these factors required to support chick motor neurons in culture, suggesting 35 that the expression of FGF receptor 1 or 2 in

embryonic motor neurons partially accounts for FGF survival effects. Given the enhanced survival of chick motor neurons cultured in the presence of both FGF-5 and bFGF, at least three distinct FGF receptors may participate in motor neuron survival, one receptor with specificity for FGF-5, one for bFGF and a third (perhaps FGF receptor 1 or 2) able to respond to both factors.

The molecular nature of the motor neuron survival promoting activity present in embryonic and adult skeletal muscle extracts that could not be immunoprecipitated by anti-FGF-5 antiserum remains undetermined. The presence in skeletal muscle of other FGF-related molecules, though not well characterized at the molecular level, is known. bFGF-like immunoreactivity has been detected in developing skeletal muscle of embryonic chick (Joseph-Silverstein et al., *J. Cell Biol.* 108:2459-2466 (1989)), rat (Gonzalez et al., *J. Cell Biol.* 110:753-765 (1990)) and rabbit (Morrow et al., *J. Clin. Invest.* 85:1816-1820 (1990)), while a molecule with bFGF-like characteristics has been detected in human embryonic and adult skeletal muscle (Vaca et al., *J. Neurosci. Res.* 23:55-63 (1989)). aFGF has been detected (immunologically) in embryonic rat skeletal muscle (Fu et al., *J. Cell. Biol.* 114:1261-1273 (1991)). aFGF mRNA has been detected by Northern analysis in embryonic cattle muscle (Alterio et al., *Biochem. Biophys. Res. Commun.* 166:1205-1212 (1990)). Nonetheless, the absence of demonstrable secretion of aFGF and bFGF renders unlikely a role for either as a target-derived neurotrophic factor for motor neurons.

A number of other candidate target-derived trophic factors for developing spinal motor neurons

have previously been proposed. Choline acetyltransferase development factor (CDF), purified from rat skeletal muscle, increased levels of choline acetyltransferase enzyme activity in embryonic rat spinal cord cultures (McManaman et al., *J. Biol. Chem.* 263:5890-5897 (1988); *J. Neurochem.* 53:1763-1771 (1989)). Although conclusive survival promoting effects of CDF on highly-enriched or purified motor neuron cultures have not been reported, CDF shares some properties with FGF-5, including (1) an inability to support the survival of chick ciliary neurons in culture, (2) apparent abundance in skeletal muscle and (3) additive effects with bFGF (McManaman et al., *supra*). Nonetheless, several differences between CDF and FGF-5, such as extent of glycosylation and heparin-binding characteristics, suggest that they are distinct entities. Identification of the exact molecular nature of CDF awaits the cloning of this factor.

A cholinergic differentiation factor for sympathetic neurons originally isolated from heart muscle conditioned medium (Fukada, K., *Proc. Natl. Acad. Sci.* 82:8795-8799 (1985)), was found upon cloning (Yamamori et al., *Science* 246:1412-1416 (1989)) to be identical to leukemia inhibitory factor (LIF). LIF exhibits a degree of functional overlap with CNTF (for review, see Patterson, P.H., *Curr. Opin. Neurobiol.* 2:94-97 (1992), including a survival effect on cultured rat motor neurons (Martinou et al., *Neuron* 8:737-744 (1992)). LIF mRNA, unlike CNTF mRNA, is present in low amounts in embryonic rat skeletal muscle (Patterson et al., *Ciba Found. Symp.* 167:125-135 (1992)). However, murine LIF is unable to act on other chick cells in culture even at high

concentrations (Rohrer, H., *Development* 114:689-698 (1992)), presumably as a result of species differences. Thus, LIF would not be expected to 5 contribute to the motor neuron survival activity of the rat muscle extracts observed in the cultures described here. To what extent LIF produced in rat skeletal muscle might promote motor neuron survival and function remains a matter of conjecture.

10 The data presented here demonstrate that FGF-5 is a major skeletal muscle-derived survival factor for cultured spinal motor neurons. Furthermore, the expression of FGF-5 in muscle and its secretory nature are compatible with the hypothesis that this protein 15 may provide target-derived trophic support to motor neurons both during embryonic development and adulthood. Other experimental systems, such as the *in vivo* administration of blocking FGF-5 antibodies during development, or the gene knockout or tissue- 20 specific overexpression of FGF-5 in transgenic animals, could be used to clarify whether FGF-5 indeed plays a role in regulating motor neuron survival *in vivo*, or perhaps partakes in the modulation of other motor neuron-related functions, such as transmitter 25 synthesis or synapse formation.

7. EXAMPLE: RECOMBINANT HUMAN FGF-5  
SUPPORTS THE SURVIVAL OF CULTURED RAT  
MOTOR NEURONS PURIFIED BY IMMUNOPANNING

30 7.1. MATERIALS AND METHODS

7.1.1. Materials

The materials used were described in Section 6.1.1 above.

7.1.2. Preparation of Panning Plates

Plates for immunopanning of motor neurons from dissociated ventral spinal cords were prepared as follows. 5 Polystyrene 10 cm diameter petri dishes were incubated overnight at 4°C with sheep anti-mouse affinity purified IgG (20 µg) in coating buffer (10 mM Tris-HCl, pH 9.5, 10 ml). The plates were washed three times with PBS and incubated for one hour at 4°C 10 with 5 ml of supernatant from MC192-producing mouse hybridoma cells (diluted 1:1 in PBS). Plates were washed again three times with PBS prior to adding cell suspensions.

15 7.1.3. Embryonic Rat Motor Neuron Cultures

Embryos were obtained from pregnant Wistar rats at day 15 of gestation following ether anesthesia and cervical dislocation of the mother. Ventral parts of the lateral spinal columns of 6 embryos were carefully 20 subdissected and cut into small pieces using forceps. The spinal columns were then treated with 0.05% trypsin in HBSS(-) for 15 minutes, washed in HBSS(-) and gently triturated 5 or 6 times in 0.1% Soybean trypsin inhibitor with a 1 ml Gilson pipette. The 25 cell suspension thus obtained was filtered through a 50 µm nylon mesh filter, and the trituration procedure was repeated with any remaining spinal cord fragments. Culture medium (bicarbonate buffered L-15 containing N1 supplements (Bottenstein et al., *Proc. Natl. Acad. Sci. USA* 76:514-517 (1979)) was added to the filtered 30 cells suspension and the suspension added to an immunopanning plate prepared as described above. Cells were allowed to attach to the plate for 1 hour at room temperature, after which the plate was gently 35 washed 8 times with 3 ml PBS to remove unattached

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cells. Bound neurons were released from the panning plates by adding 2 ml of MC192 supernatant and gently swirling for 5 minutes. Detached cells were collected 5 by aspiration, centrifuged and resuspended in culture medium prior to plating at a density of about 2000-3000 cells per well of a 4 well tissue culture dish coated with poly-ornithine and laminin (Arakawa *et al.*, *supra*). After incubating for 1 hour at 37°C in a 10 5% CO<sub>2</sub> atmosphere, neurotrophic and growth factors were added. Culture medium and factors were renewed after 24 hours in culture and thereafter every 48 hours. The total number of cells originally plated per well was determined after 4 hours. Neuron survival was 15 assessed by counting surviving neurons in culture after 5 days. Results are presented as surviving cells as a % of originally plated cells.

#### 7.2. RESULTS

20 The results are shown in Table I, below. Recombinant human FGF-5 added at a supramaximal concentration supported the survival of cultured rat motor neurons. Thus, it was concluded that recombinant human FGF-5 is able to promote similar 25 degrees of survival of both avian and mammalian motor neurons in culture over a similar concentration range.

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TABLE I

Recombinant Human FGF-5 Promotes  
Rat Motor Neuron Survival

<u>Factor</u>	<u>Concentration</u>	<u>% Survival at day 5</u>
Control	--	11 ± 5
FGF-5	100 ng/ml	57 ± 7

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The references cited above are all incorporated by reference in their entirety herein, whether 5 specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of 10 equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will 15 be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present 20 disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A method of treating a motor neuron disorder  
5 comprising administering to a subject in need of such  
treatment an effective amount of FGF-5, or a  
functional derivative thereof that supports the  
survival of motor neurons.

10 2. A method of treating a motor neuron disorder  
comprising administering to a subject in need of such  
treatment an effective amount of FGF-5, or a  
functional derivative thereof that supports the  
survival of motor neurons, in combination with an  
15 effective amount of at least one other agent that is  
capable of promoting motor neuron survival, growth, or  
differentiation.

20 3. A method according to claim 2 wherein the  
other agent is ciliary neurotrophic factor.

25 4. A method according to claim 1 or 2 wherein  
the motor neuron disorder is one which is associated  
with trauma, amyotrophic lateral sclerosis,  
progressive spinal muscular atrophy, infantile  
muscular atrophy, juvenile muscular atrophy,  
poliomyelitis, post polio syndrome, or Charcot-Marie-  
Tooth disease.

30 5. A pharmaceutical composition useful in the  
treatment of a motor neuron disorder comprising:  
(a) an amount of FGF-5 effective for treating a  
motor neuron disorder; and  
(b) a pharmaceutically acceptable carrier.

6. A pharmaceutical composition useful in the treatment of a motor neuron disorder comprising:

5 (a) an amount of a functional derivative of FGF-5 effective for treating a motor neuron disorder; and

(b) a pharmaceutically acceptable carrier.

10 7. A pharmaceutical composition useful in the treatment of a motor neuron disorder comprising the pharmaceutical composition according to claim 5 or 6, and at least one other agent that is capable of promoting motor neuron survival, growth, or differentiation.

15 8. A pharmaceutical composition according to claim 7 wherein said at least one other agent is ciliary neurotrophic factor.

20 9. A method of promoting motor neuron survival comprising exposing a motor neuron to an effective concentration of FGF-5 that is capable of promoting the survival of motor neurons.

25 10. A method according to claim 9 that is carried out *in vitro*.

11. A method according to claim 9 that is carried out *in vivo*.

30 12. A method of diagnosing a motor neuron disorder associated with an abnormal level of FGF-5 in a subject, comprising:

35 (a) measuring the level of FGF-5 in a sample from said subject; and

- 60 -

5 (b) comparing the levels of FGF-5 measured in step (a) with the level of FGF-5 in an analogous sample from a normal individual or a standard level,

10 thereby detecting an abnormality in the level of the FGF-5 in said subject, said abnormality being diagnostic of the motor neuron disorder.

15 13. A method according to claim 12 wherein the disorder is amyotrophic lateral sclerosis, progressive spinal muscular atrophy, infantile muscular atrophy, poliomyelitis, post-polio syndrome Charcot-Marie Tooth disease, nerve trauma or nerve injury.

20 14. A method for assaying a test agent for its ability to alter the binding of FGF-5 to a motor neuron, comprising:

25 (a) exposing motor neurons to detectably labeled FGF-5 under conditions wherein said labeled FGF-5 binds to the motor neurons;

(b) exposing the motor neurons of step (a) to the test agent;

(c) measuring the binding of FGF-5 to said motor neurons in the presence of said test agent;

30 (e) exposing motor neurons of step (a) to a control agent;

(f) measuring the binding of FGF-5 to said motor neurons in the presence of said control agent; and

(g) comparing the binding of the labeled FGF-5 in the presence of the test agent with the binding of labeled FGF-5 in the presence of the control agent,

wherein an increase or decrease in the binding of labeled FGF-5 to said motor neurons in the presence of said test agent indicates that said test agent alters 5 the binding of FGF-5 to motor neurons.

15. A method for assaying a test agent for its ability to enhance or inhibit the activity of FGF-5 in promoting the survival of motor neurons, comprising:

- 10 (a) exposing motor neurons to a concentration of FGF-5 effective in promoting the survival of said neurons;
- (b) exposing motor neurons of step (a) to the test agent and measuring the survival of said motor neurons;
- 15 (c) exposing motor neurons of step (a) to a control agent and measuring the survival of said control-exposed motor neurons; and
- (d) comparing the survival of said motor neurons in the presence of FGF-5 and said test agent with the survival of said motor neurons in the presence of FGF-5 and said control agent.

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1/4

FIG. 1B

E15 H

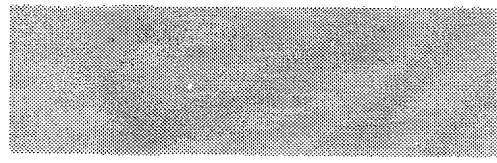
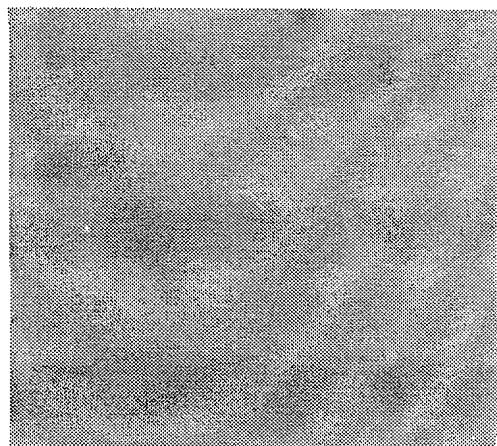


FIG. 1A

E15 NB A



28S -

<sup>18S</sup> -  
FGF-5 -

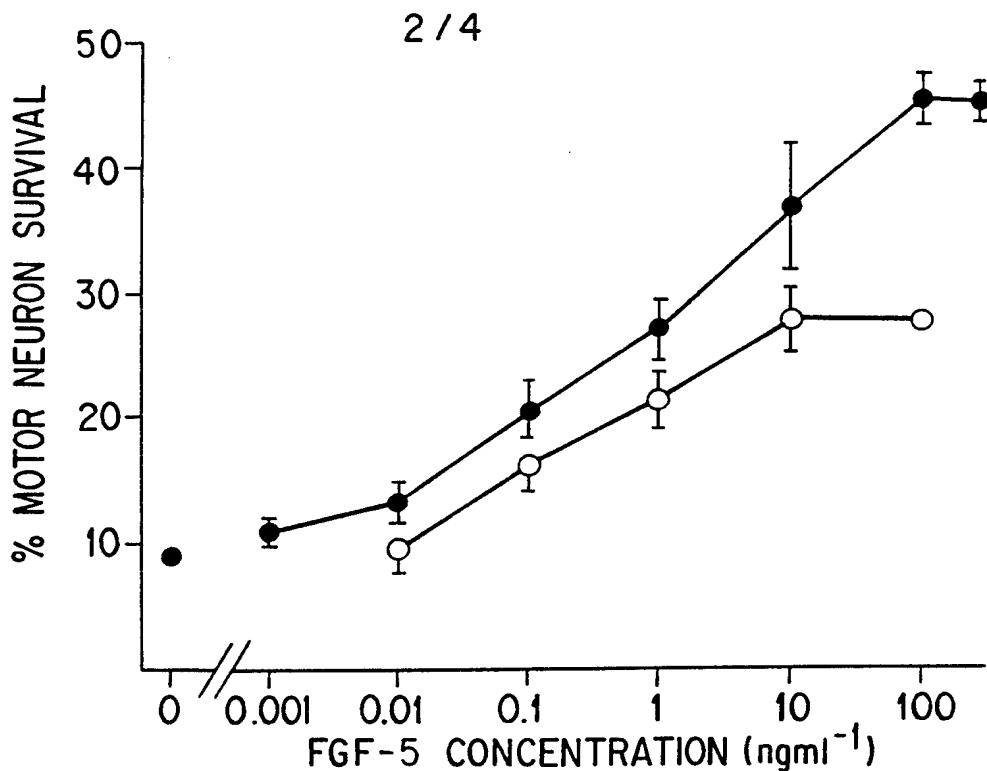


FIG. 2

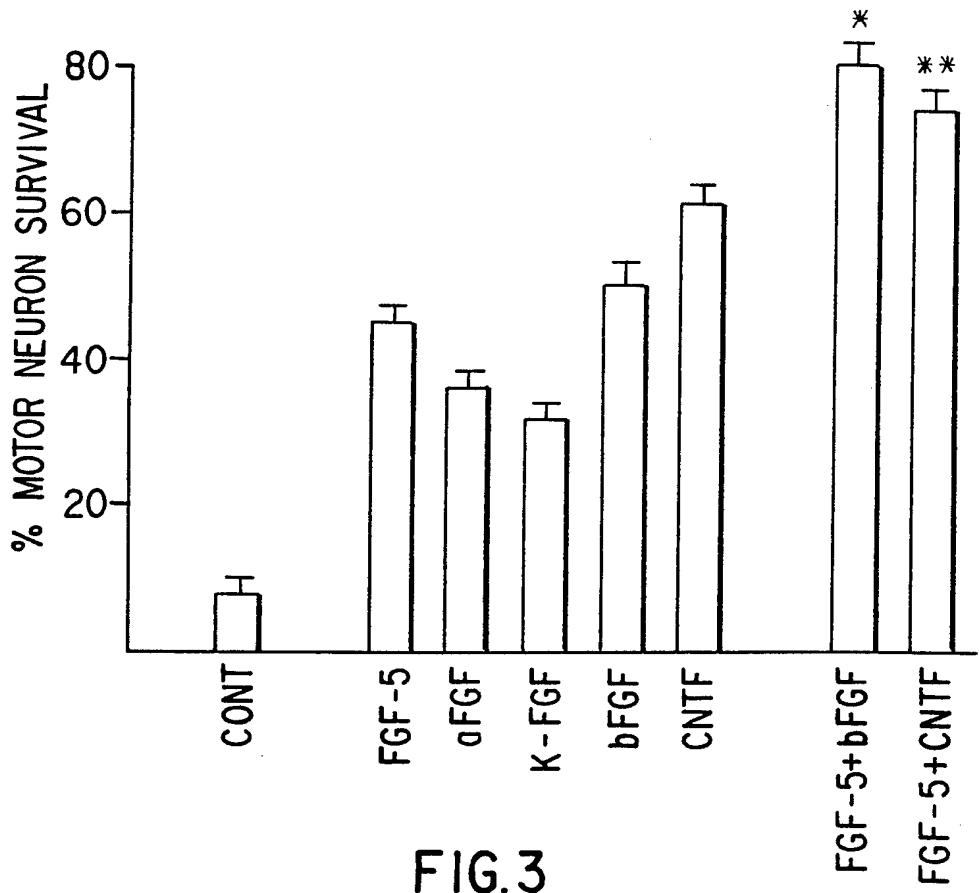
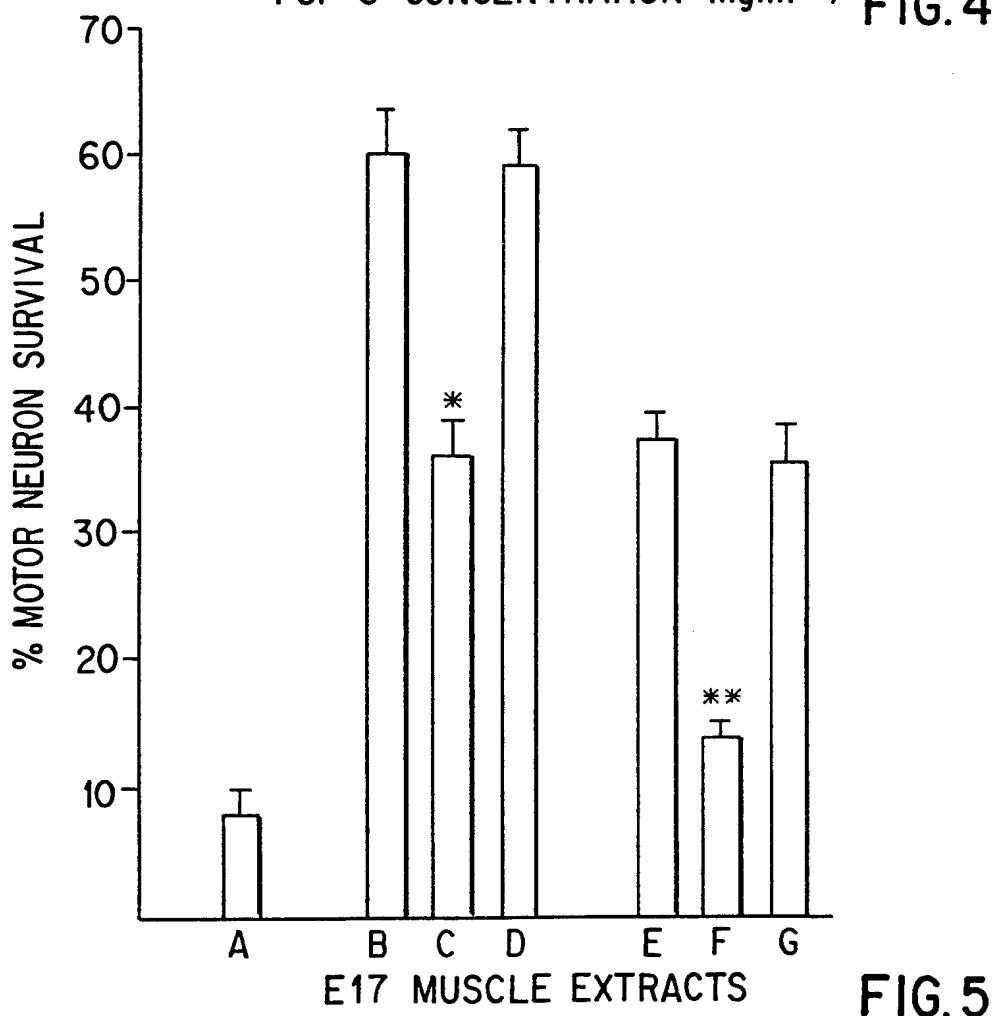
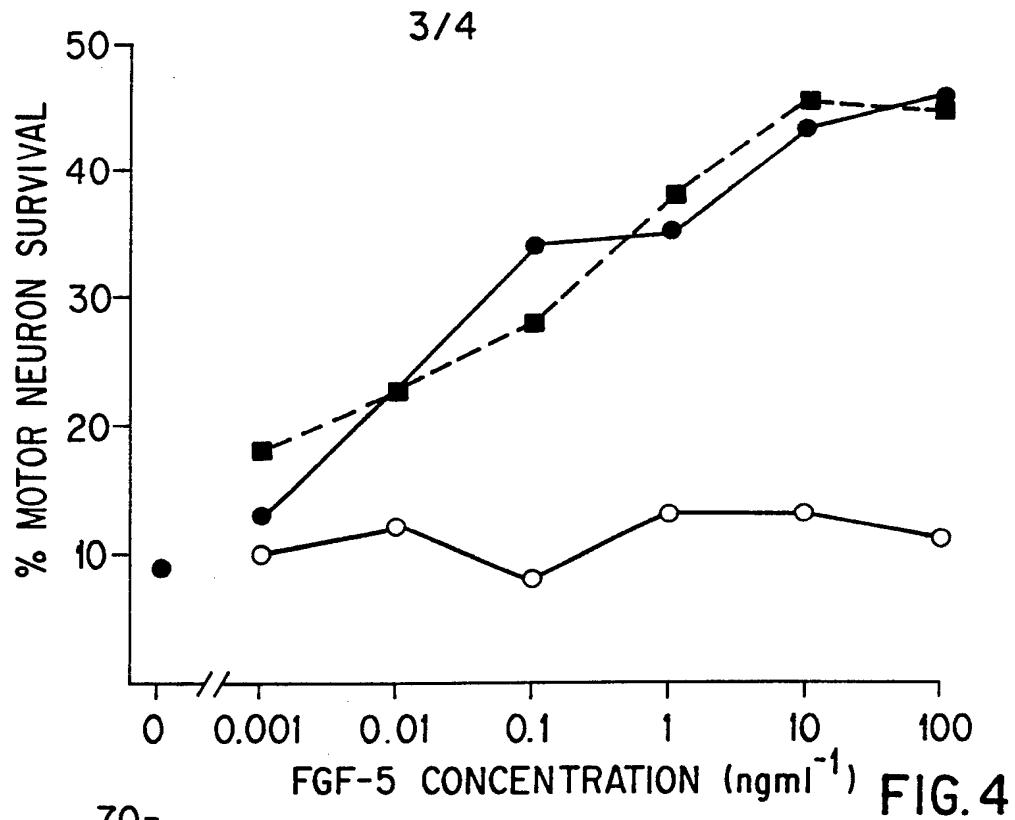


FIG. 3



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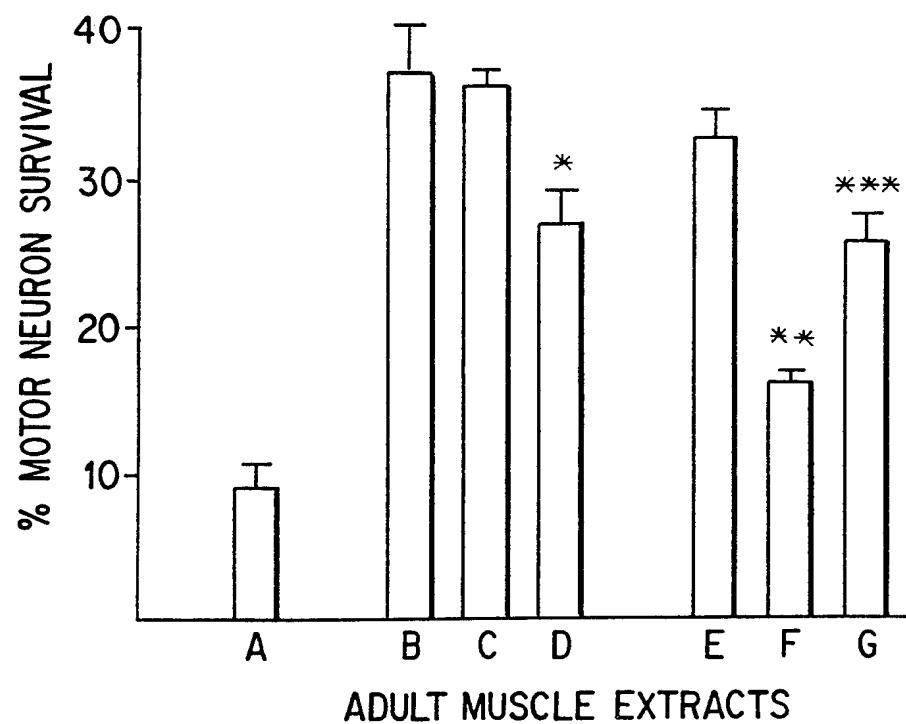


FIG. 6

## INTERNATIONAL SEARCH REPORT

Int. onal Application No  
PCT/EP 94/00764A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 A61K37/02 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR AND CELLULAR BIOLOGY vol. 8, 1988, WASHINGTON D.C., USA pages 3487 - 3495 ZHAN ET AL 'THE HUMAN FGF-5 ONCOGENE ENCODES A NOVEL PROTEIN RELATED TO FIBROBLAST GROWTH FACTORS' cited in the application see page 3487, abstract, and page 3493, paragraph 2 ---	1,5,6, 9-12,14, 15
Y	see page 3487, abstract, and page 3493, paragraph 2 ---	2-4,7,8, 13
Y	WO,A,91 04316 (MAX PLANCK INSTITUT FÜR PSYCHIATRIE) 4 April 1991 cited in the application see page 115, line 15 - page 121, line 23; claims 91-116 ---	2-4,7,8, 13
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

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Date of the actual completion of the international search  28 June 1994	Date of mailing of the international search report  12.07.94
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Sitch, W

**INTERNATIONAL SEARCH REPORT**

International Application No  
PCT/EP 94/00764

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE EUROPEAN JOURNAL OF NEUROSCIENCE vol. 6, no. 2 , 1 February 1994 , OXFORD, ENGLAND pages 244 - 252 LINDHOLM ET AL 'FIBROBLAST GROWTH FACTOR-5 PROMOTES DIFFERENTIATION OF CULTURED RAT SEPTAL CHOLINERGIC AND RAPHE SEROTONERGIC NEURONS:COMPARISON WITH THE EFFECTS OF NEUROTROPHINS' see page 244, abstract	1,5,6, 9-12,14, 15
P,Y	---	2-4,7,8, 13
P,X	NEURON vol. 10 , March 1993 , CAMBRIDGE,MA,USA pages 369 - 377 HUGHES ET AL 'EVIDENCE THAT FIBROBLAST GROWTH FACTOR 5 IS A MAJOR MUSCLE-DERIVED SURVIVAL FACTOR FOR CULTURED SPINAL MOTONEURONS' see page 369, summary	1,5,6, 9-12,14, 15
P,Y	---	2-4,7,8, 13
P,X	COMPTES RENDUS DE L'ACADEMIE DES SCIENCES.SERIE III.SCIENCES DE LA VIE vol. 316, no. 9 , September 1993 , PARIS,FRANCE pages 1158 - 1160 THOENEN ET AL 'VERS LA COMPRÉHENSION DU SOUTIEN TROPHIQUE DES NEURONES MOTEURS' see page 1158, summary	1,5,6, 9-12,14, 15
P,Y	---	2-4,7,8, 13
P,A	WO,A,94 03199 (REGENERON PHARMACEUTICALS) 17 February 1994 see claims 1-18	1-15
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/EP 94/00764

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1-4, 9 (partially), 11 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No  
PCT/EP 94/00764

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9104316	04-04-91	US-A-	5173480	22-12-92
		AU-A-	6740290	18-04-91
		CN-A-	1054099	28-08-91
		EP-A-	0448707	02-10-91
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WO-A-9403199	17-02-94	AU-B-	4995193	03-03-94
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